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Award Number: **W81XWH-09-1-0293**

TITLE: **Melanocortin and Opioid Peptide Interactions in the Modulation of Binge Alcohol Drinking**

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REPORT DATE: **April 2011**

TYPE OF REPORT: **Annual**

PREPARED FOR: **U.S. Army Medical Research and Materiel Command**
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
22-apr-2011	Annual	23 March 2010 – 22 March 2011

4. TITLE AND SUBTITLE

Melanocortin and Opioid Peptide Interactions in the Modulation of Binge Alcohol Drinking

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0293

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of North Carolina at Chapel Hill

Chapel Hill, NC 27599-3270

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland, 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Frequent binge drinking is associated with numerous negative short- and long-term consequences, including an increased risk of accidental injury, violent behavior, depression, heart disease, and type 2 diabetes. While illicit drug use and cigarette smoking both decreased significantly in the US military between the period of 1980 to 2002, heavy alcohol use increased. In fact, heavy alcohol use and binge drinking are observed in 27% of the military population. Identifying neurochemical pathways in the brain that modulate binge drinking may provide insight into pharmaceutical treatments that could protect against this dangerous behavior. Recently identified candidates for modulating binge drinking are the melanocortin (MC) peptides, such as α-melanocyte stimulating hormone (α-MSH), and the opioid peptide β-endorphin which are produced in the same brain neurons. The specific aims proposed below will test the guiding hypothesis that stimulation of MC receptor and bl ockade of opioid receptor protect against excessive binge-like alcohol drinking and i ntoxicating blood alcohol levels (BALs) in an animal model of binge drinking. The aims will also determine if MC receptor (MCR) agonists and opioid receptor antagonists interact to protect against binge-like alcohol drinking an an additive (synergistic) manner.

15. SUBJECT TERMS
Alcohol, Alcoholism, Ethanol, binge drinking, Mice, melanocortin, opioid peptide

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	21	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

INTRODUCTION: Frequent binge drinking is associated with numerous negative short- and long-term consequences, including an increased risk of accidental injury, violent behavior, depression, heart disease, and type 2 diabetes. While illicit drug use and cigarette smoking both decreased significantly in the US military between the period of 1980 to 2002, heavy alcohol use increased. In fact, heavy alcohol use and binge drinking are observed in 27% of the military population. Given that the rate of binge drinking by the civilian population is only 15%, individuals in the military are at an increased risk of regular binge drinking and thus all the health risks that are associated with this disorder. Identifying neurochemical pathways in the brain that modulate binge drinking may provide insight into pharmaceutical treatments that could protect against this dangerous behavior. Recently identified candidates for modulating binge drinking are the melanocortin (MC) peptides, such as α-melanocyte stimulating hormone (α-MSH), and the opioid peptide β-endorphin which are both cleaved from the polypeptide precursor proopiomelanocortin (POMC). The specific aims of this grant will test the guiding hypothesis that MC receptor signaling protects against excessive binge-like alcohol drinking and intoxicating blood ethanol concentrations (BECs) in an animal model of binge drinking. The aims will also determine if MC receptor (MCR) agonists and opioid receptor antagonists interact to protect against binge-like alcohol drinking in C57BL/6J mice in a supraadditive (synergistic) manner. **Specific Aims: Specific Aim 1** will test the hypothesis that binge-like alcohol drinking will be associated with a significant reduction of α-MSH levels in candidate brain regions of C57BL/6J mice, and if this reduction of α-MSH will become more robust following repeated binge episodes. This aim will also determine if repeated binge episodes promote increases of binge-like drinking in mice. **Specific Aim 2** will test the hypothesis that central infusion of MCR agonist will protect against, and MCR antagonist will augment, binge-like alcohol drinking in C57BL/6J mice via the MC-4 receptor (MC4R). Mutant mice lacking MC-3 receptor (MC3R) or MC4R will be used to determine the receptor(s) that are involved. **Specific Aim 3** will test the hypothesis MCR agonists and opioid receptor antagonists interact to protect against binge-like alcohol drinking in C57BL/6J mice in a supraadditive manner.

BODY: Experiments during the last funding cycle were directed at Tasks 1 and 2 (assessment of α-MSH and β-endorphin immunoreactivity in response to binge-like drinking episodes in C57BL/6J mice) and Tasks 7 and 8 (determine if MCR agonist MTII and opioid receptor antagonist naltrexone, when administered together, interact synergistically in block binge-like ethanol drinking in mice).

Tasks 1 : Will determine if binge-like alcohol drinking will be associated with a significant reduction of α-MSH levels in candidate brain regions of C57BL/6J mice, and if repeated binge episodes will be associated with a progressive increase of binge-like drinking. **Task 2:** Will determine if binge-like alcohol drinking will be associated with alterations of POMC, β-endorphin, or NeuN protein expression.

For these experiments, we use a 4-day “drinking in the dark” (DID) procedure to achieve binge-like ethanol drinking in C57BL/6J mice as described in the grant proposal. Briefly, homecage water bottles were replaced with a single bottle of 20% (v/v) ethanol 3-hours into the dark phase. 20% ethanol remained on the homecage for 2-hours on days 1-3 and for 4-hours on day 4 of each DID episode. Different groups of mice experienced either 1, 3, or 6 DID episodes (binges), and each 4-day DID episode was separated by 3-days in which mice were not given access to ethanol. Immediately following the 4-hours of ethanol access on day 4 of the last DID episode, tail blood samples (6 µl) were collected from all mice to determine BECs. Brains were then sliced. And processed for α-MSH, agouti-related protein (a natural MCR antagonist that is secreted in the

same terminals as α-MSH), β-endorphin, or NeuN immunoreactivity (IR). We have finished quantifying α-MSH and AgRP IR. Data depicting α-MSH IR in the lateral hypothalamus (LH), the dorsomedial hypothalamus (DMH), the paraventricular nucleus of the hypothalamus (PVN), the arcuate nucleus of the hypothalamus (ARC), and the core region of the nucleus accumbens (NAC-CORE) are presented in **Figure 1**. As is evident in each brain region, binge-like ethanol drinking was associated with a significant reduction of α-MSH IR relative to the control group of mice that drank only water (WAT), and this effect tended to become more robust with as the number of binge-like drinking episodes increased from 1 to 6. Since we have found that the MCR agonist MTII significantly blunts binge-like ethanol drinking in C57BL/6J mice (the same mice used here), the present data and our previous observations are consistent with the hypothesis that MCR signaling protects against binge-like drinking, and endogenous MCR signaling (via α-MSH) becomes compromised with repeated episodes of binge-like drinking, a pattern that could increase the size and frequency of subsequent binge-like drinking episodes and ultimately culminate in uncontrolled dependence-induced ethanol drinking. Data depicting AgRP IR in the ARC, PVN, and LH are presented in **Figure 2**. Binge-like ethanol drinking was associated with a significant increase of AgRP IR in these regions, effects which tended to become more robust with repeated episodes of binge-like drinking. Since AgRP is an endogenous MCR antagonist, increased AgRP signaling with further interfere with MCR signaling (already attenuated by reduced α-MSH signaling), further blunting this protective mechanism. We are now finishing quantification of POMC, β-endorphin, and NeuN IR. In summary, results from these experiments show that the MCR signaling system becomes compromised during bouts of binge-like drinking, and further support our hypothesis that MCR agonists will help limit excessive binge-like drinking.

Task 7 and 8: Will determine if MCR agonist and opioid receptor antagonists interact to protect against binge-like alcohol drinking in C57BL/6J mice in an additive, supraadditive (synergistic), or infraadditive interaction. To accomplish this goal, we will assess the effects of i.p. injection of MCR agonist and opioid receptor antagonist, alone and in combination, on binge-like ethanol drinking (with DID procedures) in C57BL/6J mice. Data will then be analyzed for interactions using isobolographic techniques.

We have initiated work associates with this task by first assessing the ability of the opioid receptor antagonist naltrexone and the MCR agonist MTII to protect against binge-like ethanol drinking in C57BL/6J mice. Because of the short half-life of naltrexone, we modified the DID procedure described above by assessing 2-hour binge-like ethanol drinking (rather than 4-hour) of the test day. As we previously reported, we found that when given in an i.p. injection, naltrexone dose-dependently reduced binge-like ethanol drinking and the associated BECs (**Figure 3**) in C57BL/6J mice. More specifically, the 3 and 10 mg/kg doses of naltrexone significantly reduced binge-like ethanol drinking and BECs. These data confirm our hypothesis that endogenous opioid receptor signaling positively modulates binge-like ethanol drinking in mice, and that blockade of opioid receptors protects against binge-like ethanol drinking. Thus, opioid receptor antagonists may be effective in treating uncontrolled binge drinking in humans. We also found the i.p. injection of the MCR agonist MTII before binge-like drinking dose-dependently reduced binge-like ethanol drinking and associated BECs (**Figure 4**) in C57BL/6J mice. Here, the 1, 3, and 10 mg/kg doses of MTII significantly blunted binge-like ethanol drinking during the 2-hour test. As noted above, these observations are consistent with our hypothesis that MCR agonists play a protective role against binge-like ethanol drinking. Using these data, we were able to calculate various effective doses (ED) of each drug to begin our isobolographic analyses of naltrexone and MTII interactions in the modulation of binge-like ethanol drinking. We

have started these analyses, and our data set, to date, are presented in **Figure 5**. For each drug, we calculated the ED_{30}, ED_{40}, and ED_{50} and plotted these points on 3 separate graphs (one for each ED level). Within each graph, the ED value for each drug (MTII or naltrexone) is connected by an anchor line, and each data point is surrounded by a 95% confidence interval. Using these base figures, we have begun to determine the nature of the interactions between naltrexone and MTII. In an initial study, we performed a dose response study in which various doses of MTII were given in combination with the ED_{30} dose of naltrexone (0.82 mg/kg). The triangular data points in each graph reflect the combined administration of MTII and naltrexone. In all 3 graphs, the data point representing binge-like drinking following co-administration of naltrexone and MTII did not significantly deviate from the anchor line (i.e., data points were within the 95% confidence intervals), indicating that MTII and naltrexone produce additive effects on binge-like ethanol drinking. Data points significantly below the anchor line would support a supraadditive (synergistic) interaction between the drug, and data point significantly above the anchor line would indicate subtractive interactions between drugs. We continue to test different dose combinations to gain a more accurate picture of the interactions between MTII and naltrexone in the modulation of binge-like ethanol drinking.

KEY RESEARCH ACCOMPLISHMENTS: A list of key research accomplishments achieved during the 2nd budget year of this grant are as follows:

- Established that repeated bouts of binge-like ethanol drinking are associated significant attenuation of α-MSH immunoreactivity in brain regions implicated in the modulation of neurobiological responses to ethanol.

- Established that repeated bouts of binge-like ethanol drinking are associated with significant increases of agouti-related protein (AgRP; a natural MCR antagonist) in brain regions implicated in the modulation of neurobiological responses to ethanol.

- Established that the opioid receptor antagonist naltrexone and the MCR agonist MTII dose-dependently protect against binge-like ethanol drinking in mice.

- Began an initial assessment of the interactions of naltrexone and MTII, which suggest that combined administration of these drugs protect against binge-like ethanol drinking in C57BL/6J mice in an additive interaction. Additional dose combinations will be probed to further characterize this interaction.

REPORTABLE OUTCOMES: The following is a list of publications and a published abstract that have been supported by this grant during the 2nd budget year:

PUBLICATONS

1. Hayes, D. M., Fee, J. R., McCown, T. J., Knapp, D. J., Breese, G. R., Cubero, I., Carvajal, F., Lerma-Cabrera, J. M., Navarro, M., & Thiele, T. E. (in press). Neuropeptide Y signaling modulates the expression of ethanol-induced behavioral sensitization in mice. *Addiction Biology*.

2. Navarro, M., Lerma-Cabrera, J. M., Carvajal, F., Lowery, E. G., Cubero, I., & Thiele, T. E. (in press). Assessment of voluntary ethanol consumption and the effects of a melanocortin (MC) receptor agonist on ethanol intake in mutant C57BL/6J mice lacking

the MC-4 receptor. *Alcoholism: Clinical & Experimental Research, 35,* 1-9. [see reprint below].

3. Lyons, A. M. & Thiele, T. E. (2010). Neuropeptide Y conjugated to saporin alters anxiety-like behavior when injected into the central nucleus of the amygdala or basomedial hypothalamus in BALB/cJ mice. *Peptides*, 31, 2193-2199.

CONFERENCE PRESENTATION

1. Navarro, M., Lerma-Cabrera, J. M., Carvajal, F., Lowery, E. G., Cubero, I., & Thiele, T. E. (2010). Central, but not peripheral, administration of melanocortin (MC) receptor agonists require the MC-4 receptor to reduce ethanol intake. *Alcoholism: Clinical & Experimental Research, 34,* 15A.

CONCLUSIONS: We have made significant progress towards the goals of this research proposal during the first and second years of funding. Consistent with our hypothesis, we have found that repeated bouts of binge-like drinking were associated with a progressive increase on the amount of ethanol consumed (last progress report), which is associated with a significant decrease in central α-MSH levels and significant increases of AgRP levels. We are currently assessing the levels of central of POMC and β-endorphin. We have found that the MCR agonist MTII protects against excessive ethanol drinking by signaling through the MC4R (last progress report; also see appended publication), and established that the opioid receptor antagonist naltrexone and MCR agonist MTII protects against binge-like ethanol drinking in a dose-dependent manner. More recently, we have begun our isobolographic analyses of naltrexone and MTII interactions in the modulation of binge-like ethanol drinking and the current data set suggest an additive interaction between drugs. So what does this mean? These results have important implications for possible pharmacological medical treatment of binge drinking in the human population. Specifically, melanocortin receptor agonists aimed at the MC4R, as well a opioid receptor antagonist, may prevent binge drinking in at-risk individuals, and thus protect these people from the negative behavioral and biological consequences of regular binge drinking. Importantly, preventing frequent binge drinking will reduce the risk of future alcohol abuse disorders and dependence. These findings may be considered of high relevance to the U.S. military given the high prevalence of binge drinking in the military population.

APPENDICES:

- Figures 1-5. In figures, * indicates significant differences from the control group condition at the $p < 0.05$ level. ** indicates significant differences from the control group condition at the $p < 0.01$ level.

- 1 published paper that was supported by this grant.

Figure 1

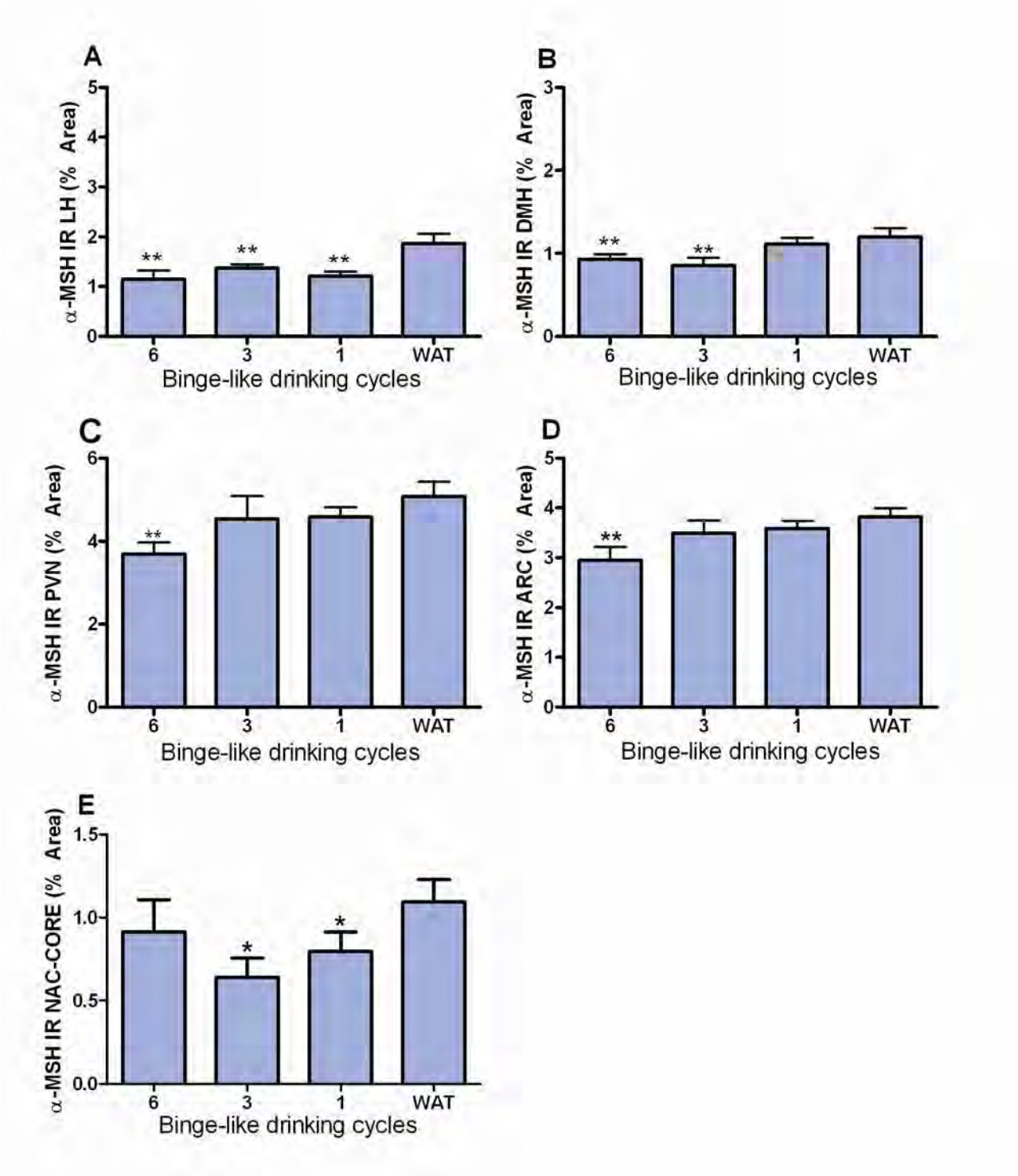

A
α-MSH IR LH (% Area)
5
4
3
2
1
0
**
**
**
6
3
1
WAT
Binge-like drinking cycles
B
α-MSH IR DMH (% Area)
3
2
1
0
**
**
6
3
1
WAT
Binge-like drinking cycles
C
α-MSH IR PVN (% Area)
6
4
2
0
**
6
3
1
WAT
Binge-like drinking cycles
D
α-MSH IR ARC (% Area)
5
4
3
2
1
0
**
6
3
1
WAT
Binge-like drinking cycles
E
α-MSH IR NAC-CORE (% Area)
1.5
1.0
0.5
0.0
*
*
6
3
1
WAT
Binge-like drinking cycles

Figure 2

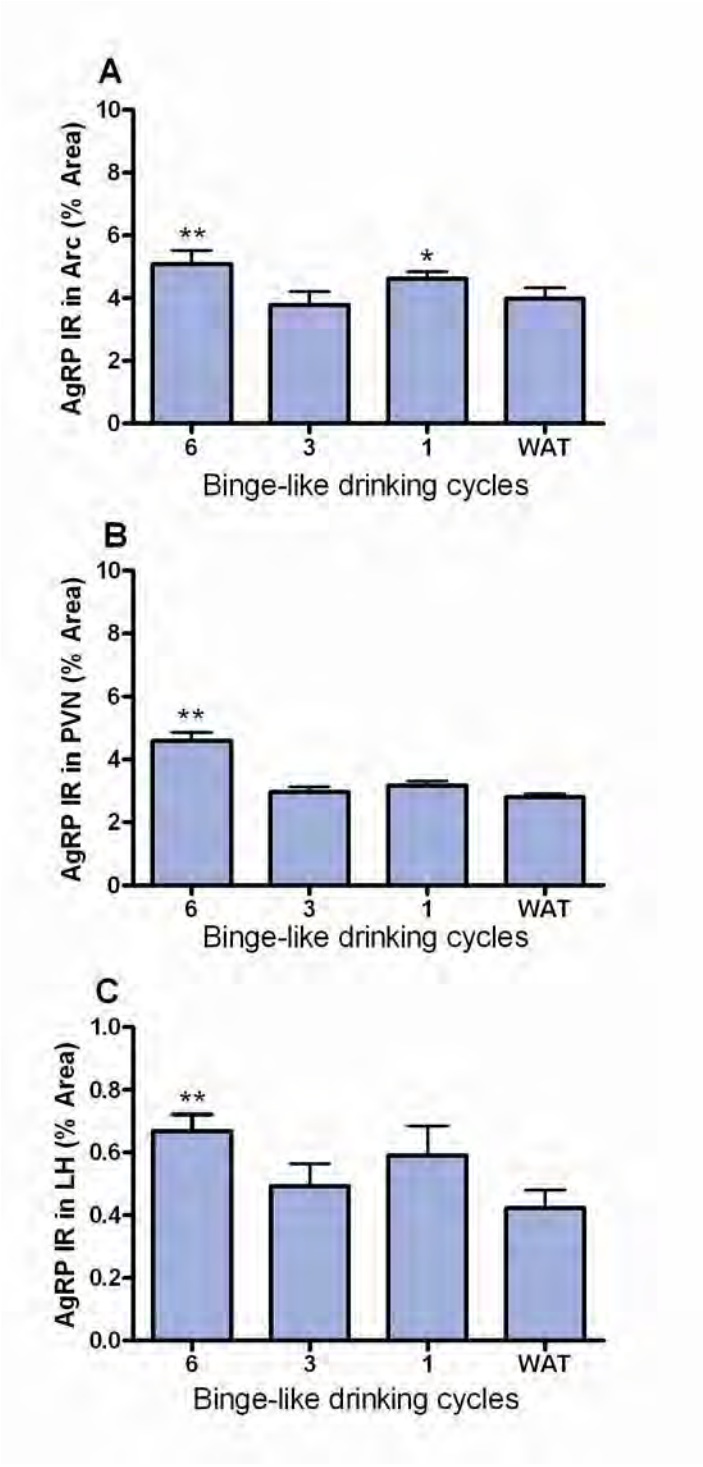

A
AgRP IR in Arc (% Area)
10
8
6
4
2
0
**
*
6
3
1
WAT
Binge-like drinking cycles
B
AgRP IR in PVN (% Area)
10
8
6
4
2
0
**
6
3
1
WAT
Binge-like drinking cycles
C
AgRP IR in LH (% Area)
1.0
0.8
0.6
0.4
0.2
0.0
**
6
3
1
WAT
Binge-like drinking cycles

Figure 3

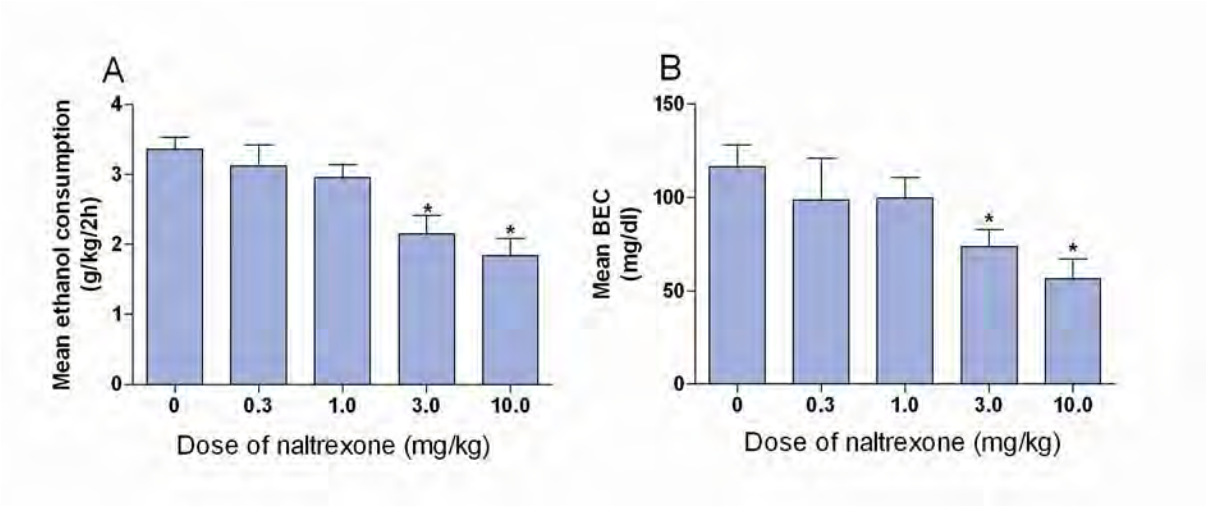

A
Mean ethanol consumption (g/kg/2h)
4
3
2
1
0
0
0.3
1.0
3.0
10.0
Dose of naltrexone (mg/kg)
B
Mean BEC (mg/dl)
150
100
50
0
0
0.3
1.0
3.0
10.0
Dose of naltrexone (mg/kg)

Figure 4

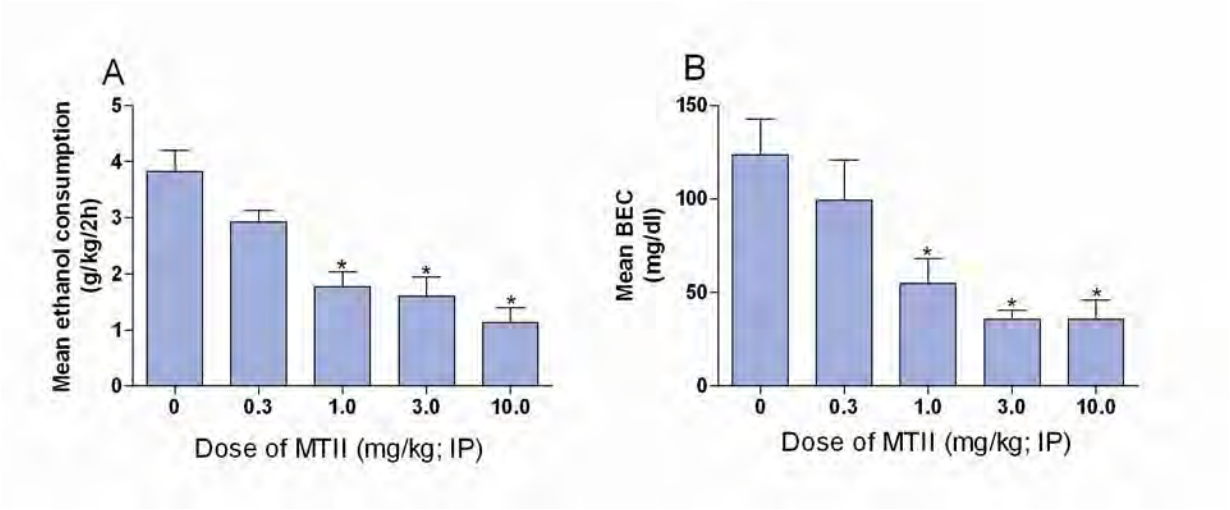
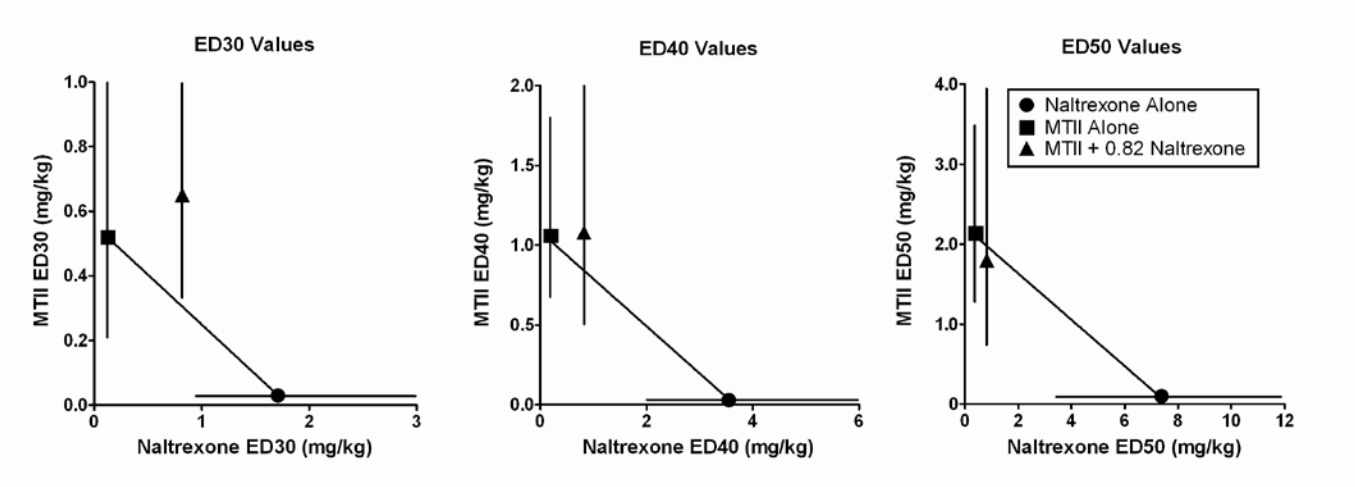

A
Mean ethanol consumption (g/kg/2h)
5
4
3
2
1
0
0
0.3
1.0
3.0
10.0
Dose of MTII (mg/kg; IP)
B
Mean BEC (mg/dl)
150
100
50
0
0
0.3
1.0
3.0
10.0
Dose of MTII (mg/kg; IP)

Figure 5


ED30 Values
MTII ED30 (mg/kg)
Naltrexone ED30 (mg/kg)
ED40 Values
MTII ED40 (mg/kg)
Naltrexone ED40 (mg/kg)
ED50 Values
MTII ED50 (mg/kg)
Naltrexone ED50 (mg/kg)
Naltrexone Alone
MTII Alone
MTII + 0.82 Naltrexone

Assessment of Voluntary Ethanol Consumption and the Effects of a Melanocortin (MC) Receptor Agonist on Ethanol Intake in Mutant C57BL/6J Mice Lacking the MC-4 Receptor

Montserrat Navarro, Jose M. Lerma-Cabrera, Francisca Carvajal, Emily G. Lowery, Inmaculada Cubero, and Todd E. Thiele

Background: The melanocortin (MC) system is composed of peptides that are cleaved from the polypeptide precursor proopiomelanocortin (POMC). Recent evidence shows that chronic exposure to ethanol significantly blunts central MC peptide immunoreactivity and MC receptor (MCR) agonists protect against high ethanol intake characteristic of C57BL/6J mice. Here, we assessed the role of the MC-4 receptor (MC4R) in voluntary ethanol intake and in modulating the effects of the nonselective MCR agonist melanotan-II (MTII) on ethanol consumption.

Methods: To assess the role of the MC4R, MC4R knockout ($Mc4r^{-/-}$) and littermate wild-type ($Mc4r^{+/+}$) mice on a C57BL/6J background were used. Voluntary ethanol (3, 5, 8, 10, 15, and 20%, v/v) and water intake were assessed using standard two-bottle procedures. In separate experiments, $Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice were given intracerebroventricular (i.c.v.) infusion of MTII (0, 0.5, or 1.0 μg/1 μl) or intraperitoneal (i.p.) injection of MTII (0 or 5 mg/kg/5 ml). The effects of MTII (0 or 0.5 μg/1 μl, i.c.v.) on 10% sucrose and 0.15% saccharin intake were assessed in C57BL/6J mice.

Results: $Mc4r^{-/-}$ mice showed normal consumption of ethanol over all concentrations tested. I.c.v. infusion of MTII significantly reduced ethanol drinking in $Mc4r^{+/+}$ mice, but failed to influence ethanol intake in $Mc4r^{-/-}$ mice. When administered in an i.p. injection, MTII significantly reduced ethanol drinking in both $Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice. MTII attenuated consumption of caloric (ethanol, sucrose, and food) and noncaloric (saccharin) reinforcers.

Conclusions: When given centrally, the MCR agonist MTII reduced ethanol drinking by signaling through the MC4R. On the other hand, MTII-induced reduction of ethanol drinking did not require the MC4R when administered peripherally. Together, the present observations show that the MC4R is necessary for the central actions of MCR agonists on ethanol drinking and that MTII blunts the consumption natural reinforcers, regardless of caloric content, in addition to ethanol.

Key Words: Ethanol Consumption, Melanocortin, MC-3 Receptor, MC-4 Receptor, C57BL/6J, Food Intake.

THE MELANOCORTIN (MC) SYSTEM is composed of peptides that are cleaved from the polypeptide precursor proopiomelanocortin (POMC). Central MC peptides are produced by neurons within the hypothalamic arcuate nucleus, the nucleus of the solitary tract, and the medulla (Crine et al., 1978; Dores et al., 1986; Hadley and Haskell-Luevano, 1999; Jacobowitz and O'Donohue, 1978; O'Donohue and Dorsa, 1982) and include adrenocorticotropic hormone, α-melanocyte-stimulating hormone (α-MSH), β-MSH, and γ-MSH (Hadley and Haskell-Luevano, 1999). Because of a lack of critical dibasic site, β-MSH is not processed in rodent brain (Pritchard et al., 2002). MC neuropeptides act through at least five receptor subtypes, namely MC-1 receptor (MC1R), MC2R, MC3R, MC4R, and MC5R, all of which couple to heterotrimeric Gs-proteins that stimulate adenylyl cyclase activity (Hadley and Haskell-Luevano, 1999). MC receptors (MCRs) in the rodent brain are primarily comprised of the MC3R and MC4R subtypes (Adan and Gispen, 1997), whereas MC1R and MC5R are detected at low levels and only in limited brain regions while the MC2R is expressed primarily in the adrenal cortex (Adan and Gispen, 1997; Barrett et al., 1994; Xia et al., 1995).

It is well established that MCR signaling is involved in the regulation of appetite and energy homeostasis (Gao and Horvath, 2008). A growing literature suggests that there is

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Received for publication August 17, 2010; accepted November 12, 2010.

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DOI: 10.1111/j.1530-0277.2011.01438.x

overlapping peptide control of ethanol consumption and feeding behavior (Thiele et al., 2003, 2004), which includes recent evidence that MCR signaling modulates neurobiological responses to ethanol. MCR and α-MSH expression have been identified in brain regions that modulate the reinforcing properties of ethanol, including the nucleus accumbens (NAc), ventral tegmental area, the bed nucleus of the stria terminalis, and amygdala (Bloch et al., 1979; Dube et al., 1978; Jacobowitz and O'Donohue, 1978; O'Donohue and Jacobowitz, 1980; O'Donohue et al., 1979; Yamazoe et al., 1984). Genetic evidence and pharmacological evidence implicate the MC system in the control of voluntary ethanol consumption. Relative to ANA (Alko, Nonalcohol) rats, AA (Alko, Ethanol) rats, selectively bred for high ethanol intake, have significantly lower levels of MC3R in the shell of the NAc, and significantly higher levels of MC3R in the paraventricular, arcuate, and ventromedial nuclei of the hypothalamus. AA rats also have high levels of MC4R in the ventromedial nucleus of the hypothalamus (Lindblom et al., 2002). These data suggest that the high ethanol drinking by AA rats may be mediated, in part, by alterations in central MCR signaling. Consistent with this hypothesis, intracerebroventricular (i.c.v.) infusion of the potent nonselective MCR agonist melanotan-II (MTII) significantly reduced voluntary ethanol drinking by AA rats (Ploj et al., 2002). Similarly, we have found that i.c.v. infusion of MTII and a selective MC4R agonist reduced ethanol drinking (Navarro et al., 2003, 2005), while ventricular infusion of the nonselective MCR antagonist agouti-related protein (AgRP) significantly increased ethanol drinking (Navarro et al., 2005), by high-ethanol-drinking C57BL/6J mice. Consistent with pharmacological data, genetic deletion of endogenous AgRP reduced ethanol-reinforced lever pressing and binge-like ethanol drinking in C57BL/6J (Navarro et al., 2009). Ethanol also has direct effects of central MC and AgRP activity. Thus, chronic exposure to ethanol significantly reduced α-MSH immunoreactivity in specific regions of the rat brain (Navarro et al., 2008), and acute administration of ethanol significantly increased AgRP immunoreactivity in the arcuate nucleus of the hypothalamus of C57BL/6J mice (Cubero et al., 2010).

The MCRs that modulate neurobiological responses to ethanol remain unclear. With respect to ethanol consumption, we found that MTII was similarly effective at reducing ethanol intake in both MC3R knock-out ($Mc3r^{-/-}$) and littermate wild-type ($Mc3r^{+/+}$) mice (Navarro et al., 2005). Furthermore, i.c.v. infusion of the highly selective MC4R agonist, cyclo(NH-CH_2-CH_2-CO-His-D-Phe-Arg-Trp-Glu)-NH_2, dose-dependently reduced ethanol drinking by C57BL/6J mice (Navarro et al., 2005). These data suggest that the MC3R does not modulate MCR agonist-induced reductions of ethanol consumption and that the MC4R is a likely candidate. The first goal of the present report was to directly assess the role of the MC4R. To this end, we examined voluntary ethanol consumption and the effects of centrally and peripherally administered MTII on ethanol intake, in $Mc4r^{-/-}$ and littermate $Mc4r^{+/+}$ mice. The second goal was to further characterize the effects of MTII on consumption of other caloric (food and sucrose) and noncaloric (saccharin) reinforcers.

MATERIALS AND METHODS

Animals

The generation of $Mc4r^{-/-}$ mice has been described elsewhere (Huszar et al., 1997). The $Mc4r^{-/-}$ mice were originally derived on a mixed 129/SvJ × C57BL/6J genetic background and show increased body weight and feeding behavior beginning at about 3-4 months of age (Huszar et al., 1997; Ste Marie et al., 2000). For the present work, we backcrossed $Mc4r^{-/-}$ mice to a C57BL/6J genetic background for 8 generations. Despite the lack of the MC4R, $Mc4r^{-/-}$ mice show normal brain expression of MC3R mRNA (Rowland et al., 2010). Littermate knockout and wild-type mice were used, and approximately equal numbers of male and female mice were used in each treatment condition. Because we have previously found no sex differences in the effect of MTII on ethanol consumption in C57BL/6J mice (Navarro et al., 2005), and because of low numbers of male and female mice within each treatment condition, sex was not included as a factor in analyses described later. The genetic status of all mice was determined using polymerase chain reaction (PCR) procedures, and mice were approximately 6 weeks of age at the beginning of experiments. We also used male C57BL/6J mice that were purchased at 6 weeks of age from Jackson Laboratory (Bar Harbor, ME). Mice were individually housed in polypropylene cages with corncob bedding and had ad libitum access to water and standard rodent chow (Tekland, Madison, WI) throughout each experiment. The colony room was maintained at approximately 22°C with a reverse 12:12 hours light:dark cycle with lights off at 10:00 AM. All procedures used in this study were in compliance with the National Institute of Health guidelines, and all protocols were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Experiment 1: Two-Bottle Consumption of Ethanol, Sucrose, Saccharin, and Water

$Mc4r^{-/-}$ ($n = 8$) and $Mc4r^{+/+}$ ($n = 9$) mice were tested for voluntary ethanol consumption using a homecage 2-bottle choice procedure. Over 4 days, mice were given 24-hour access to 2-bottles on their homecage, one containing tap water and the other containing a 3% (v/v) ethanol solution. The concentrations of ethanol were then increased to 5, 8, 10, 15, and 20% every 4 days. The positions of the bottles were alternated every 2 days to control for position preferences. Each drinking bottle was weighed every 2 days, and body weights were recorded every 4 days. An empty cage was used for the placement of dummy bottles (1 ethanol and 1 water), and fluid lost from each of these bottles was subtracted off the consumption totals as a control for fluid spillage. A separate set of $Mc4r^{-/-}$ ($n = 12$) and $Mc4r^{+/+}$ ($n = 12$) mice were tested for voluntary consumption of 0.15% (w/v) saccharin solution versus water followed by 10% (w/v) sucrose solution versus water in a two-bottle test. Mice were given access to each sweet solution for 2 days.

Experiment 2: Ethanol Consumption Following Central Infusion of the MCR Agonist MTII

Mice were anesthetized with a cocktail of ketamine (117 mg/kg) and xylazine (7.92 mg/kg) and surgically implanted with a 26-gauge guide cannula (Plastic One, Roanoke, VA) aimed at the left lateral ventricle, with the following stereotaxic coordinates: 0.2 mm posterior to bregma, 1.0 mm lateral to the midline, and 2.3 mm ventral to the surface. Mice were allowed to recover for approximately 2 weeks before experimental procedures were initiated. After experimental

procedures, cannula placement was verified histologically. I.c.v. infusions were given in a 1.0 μl volume over a 1-minute period using a 33-gauge injector needle that extended 0.5 mm beyond that guide cannula. Compounds were administered manually with a 1-μl Hamilton syringe. The injectors were left in place for an additional 1 minute to allow for drug diffusion and to minimize vertical capillary action along the injector tract when it was removed.

After recovery from surgery, animals received 5 days of habituation to 24 hour, 2-bottle consumption with one bottle containing water and the second bottle containing a solution of 10% (v/v) ethanol. Following habituation to 2-bottle drinking, mice from each genotype were distributed into groups that were equated based on ethanol consumption measured during the last 3 days of baseline. On the test day, mice were weighed, and ethanol, water, and food were removed from their cages 2 hours before the beginning of the dark cycle. Approximately 30- to 15-minutes before the beginning of the dark cycle, mice were given i.c.v. infusions of a 0.5 ($Mc4r^{-/-}$ mice, $n = 11$; $Mc4r^{+/+}$ mice, $n = 8$)- or 1 ($Mc4r^{-/-}$ mice, $n = 6$; $Mc4r^{+/+}$ mice, $n = 5$)-μg dose of MTII (Bachem, Torrance, CA) dissolved 0.9% saline, or an equal volume of 0.9% saline ($Mc4r^{-/-}$ mice, $n = 8$; $Mc4r^{+/+}$ mice, $n = 7$). We have previously found that the 1-μg dose of MTII was effective in reducing ethanol intake in C57BL/6J mice (Navarro et al., 2003, 2005). We chose MTII as we previously assessed the effects of MTII in $Mc3r^{-/-}$ mice (Navarro et al., 2005) and could thus make direct comparisons between studies. The 10% ethanol solution, water, and food were returned immediately before the dark cycle. Intake measures were recorded 6 hours later.

Experiment 3: Ethanol Consumption Following Peripheral Administration of the MCR Agonist MTII

$Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice received 5 days of habituation to 24 hour, 2-bottle consumption with 1 bottle containing water and the second bottle containing a solution of 10% (v/v) ethanol. Following habituation to 2-bottle drinking, mice from each genotype were distributed into groups that were equated based on ethanol consumption measured during the last 3 days of baseline. On the test day, mice were weighed, and ethanol, water, and food were removed from their cages 2 hours before the beginning of the dark cycle. Approximately 30- to 15-minutes before the beginning of the dark cycle, mice were given an intraperitoneal (i.p.) injection of a 5 mg/kg dose of MTII dissolved in 0.9% saline ($Mc4r^{-/-}$ mice, $n = 15$; $Mc4r^{+/+}$ mice, $n = 14$) or an equal volume of 0.9% saline given in a 5 ml/kg volume ($Mc4r^{-/-}$ mice, $n = 15$; $Mc4r^{+/+}$ mice, $n = 15$). We chose the 5 mg/kg dose of MTII because it falls between doses (2 and 10 mg/kg) that have been shown to effectively attenuate feeding behavior (Chen et al., 2000; Choi et al., 2003). The 10% ethanol solution, water, and food were returned immediately before the dark cycle. Intake measures were recorded 6 hours later.

Experiments 4 and 5: Sucrose and Saccharin Solution Consumption Following Central Infusion of the MCR Agonist MTII

Surgery for cannula placement and i.c.v. infusion procedures were the same as described in Experiment 2. After recovery from surgery, animals received 5 days of habituation to 24 hour, 2-bottle consumption with one bottle containing water and the second bottle containing a solution of 10% (w/v) sucrose (Experiment 4) or 0.15% (w/v) saccharin (Experiment 5). Following habituation to 2-bottle drinking, mice from each genotype were distributed into groups that were equated based on sweet solution consumption measured during the last 3 days of baseline. On the test day, mice were weighed, and sweet solution, water, and food were removed from their cages 2 hours before the beginning of the dark cycle. Approximately 30 to 15 minutes before the beginning of the dark cycle, mice were given i.c.v. infusions of a 0.5-μg dose of MTII dissolved 0.9% saline (Experiment 4, $n = 8$; Experiment 5, $n = 12$) or an equal volume of 0.9% saline (Experiment 4, $n = 7$; Experiment 5, $n = 12$). The sweet solution, water, and food were returned immediately before the dark cycle. Intake measures were recorded 6 hours later.

Data Analyses

To obtain a measure that corrected for individual differences in body weight, grams of ethanol or food and milliliters of water or sweet solution consumed per kilogram of body weight were calculated. Ethanol preference ratios were also calculated by dividing the volume of ethanol consumed by total fluid (ethanol + water) consumption. Ethanol consumption data from Experiment 1 were analyzed with a 2 x 6 (genotype $\times$ ethanol concentration) repeated-measures analysis of variance (ANOVA), and saccharin and sucrose consumption data were analyzed with 2×2 (genotype $\times$ days) repeated-measures ANOVAs. Data from Experiments 2 and 3 were analyzed with two-way 2×3 (genotype $\times$ MTII dose) mixed-factor ANOVAs. Finally, data from Experiments 4 and 5 were analyzed using one-way (dose) ANOVAs. Tukey's tests were used for post hoc analyses. All data are presented as means $\pm$ SEM, and the level of significance was set at $p < 0.05$ in all cases.

RESULTS

Experiment 1: Two-Bottle Consumption of Ethanol and Water

Data showing 24-hour voluntary consumption of ethanol and water and ethanol preference ratios in $Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice during 2-bottle testing are presented in Fig. 1. A repeated-measures ANOVA performed on ethanol consumption data revealed a significant main effect of ethanol concentration [$F(5,75) = 59.149$; $p = 0.001$], reflecting the increase in g/kg of ethanol consumed as the concentration of ethanol was increased over the course of the experiment (Fig. 1A). No other effects were statistically significant. A repeated-measures ANOVA performed on water consumption data revealed a significant main effect of ethanol concentration phase [$F(5,75) = 23.685$; $p = 0.001$], reflecting the greater consumption of water as the concentration of ethanol was increased. Interestingly, there was a significant main effect of genotype [$F(1,15) = 5.473$; $p = 0.034$], as $Mc4r^{+/+}$ mice (71.98 $\pm$ 5.24 ml/kg/24-h) drank significantly more water than $Mc4r^{-/-}$ mice (54.11 $\pm$ 5.56 ml/kg/24-h) over the course of the experiment (Fig. 1B). No other effects related to the water data were statistically significant. A repeated-measures ANOVA performed on ethanol preference ratio data revealed a significant main effect of ethanol concentration phase [$F(5,75) = 26.831$; $p = 0.001$], reflecting the reduced preference for ethanol solution relative to water as the concentration of ethanol was increased (Fig. 1C). Finally, a repeated-measures ANOVA comparing body weight data at each phase of the experiment revealed that there were no significant differences in body weight between $Mc4r^{+/+}$ mice (20.40 $\pm$ 1.09 g average over the course of the experiment) and $Mc4r^{-/-}$ mice (23.23 $\pm$ 1.11 g average over the course of the experiment).

A repeated-measures ANOVAs performed on saccharin consumption data revealed a significant effects of days [$F(1,22) = 8.627$; $p = 0.008$], reflecting increased consumption of

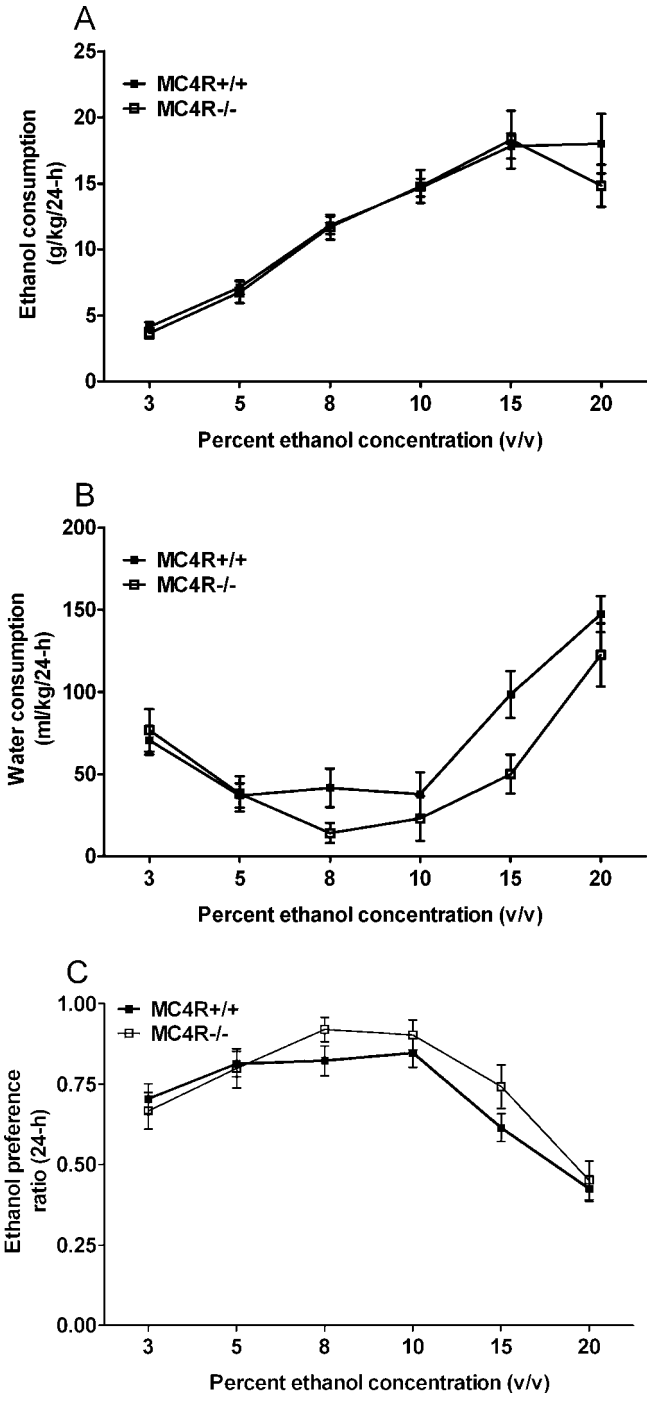

Fig. 1. Voluntary consumption of 3, 5, 8, 10, 15, and 20% (v/v) ethanol (panel **A**), water during access to different concentrations of ethanol (panel **B**), and ethanol preference ratios at each concentration of ethanol (panel **C**) in $Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice in the two-bottle testing study (Experiment 1). All values are means ± SEM. $Mc4r^{+/+}$ mice drank significantly more water than $Mc4r^{-/-}$ mice over the course of the experiment as revealed by a significant main effect of genotype ($p = 0.001$).

0.15% saccharin over days. However, $Mc4r^{-/-}$ mice (289.66 ± 41.32 ml/kg/d) and $Mc4r^{+/+}$ mice (302.51 ± 35.24 ml/kg/d) did not differ significantly in the volume of saccharin solution consumed, nor were there any genotype differences in water intake during access to saccharin. Similarly, a repeated-measures ANOVAs performed on sucrose consumption data revealed a significant effect of days [$F(1,22) = 80.103$; $p = 0.001$], reflecting increased consumption of 10% sucrose over days. $Mc4r^{-/-}$ mice (390.50 ± 13.29 ml/kg/d) and $Mc4r^{+/+}$ mice (388.00 ± 26.44 ml/kg/d) did not differ significantly in the volume of sucrose solution consumed, nor were there any genotype differences in water intake during access to sucrose.

Experiment 2: Ethanol Consumption Following Central Infusion of the MCR Agonist MTII

Data showing 6-hour consumption measures following i.c.v. infusion of MTII in the $Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice are presented in Fig. 2A–C. A two-way ANOVA performed on ethanol consumption data revealed a significant interaction effect between genotype and MTII dose [$F(2,39) = 3.739$; $p = 0.033$], but the genotype and MTII dose main effects were not significant. Post hoc tests showed that while each dose of MTII significantly reduced ethanol intake relative to control infusion in $Mc4r^{+/+}$ mice, neither dose tested altered ethanol intake in the $Mc4r^{-/-}$ mice (Fig. 2A). A two-way ANOVA performed on food intake data revealed a main effect of genotype [$F(1,39) = 6.854$; $p = 0.013$] and a significant interaction between genotype and MTII dose [$F(2,39) = 6.747$; $p = 0.003$] (Fig. 2B). Post hoc tests showed that while MTII was ineffective in $Mc4r^{-/-}$ mice, each dose of the agonist tested significantly reduced food intake (relative to vehicle treatment) in the $Mc4r^{+/+}$ mice. A two-way ANOVA performed on water intake data showed a significant interaction between genotype and MTII dose [$F(2,39) = 4.147$; $p = 0.023$], but the main effects were not statistically significant (Fig. 2C). Despite the significant interaction effect, post hoc tests revealed that MTII did not significantly alter water drinking relative to the vehicle treatment in either $Mc4r^{-/-}$ or $Mc4r^{+/+}$ mice. A two-way ANOVA performed on ethanol preference ratio data failed to show any significant effects (data not shown). Finally, a two-way ANOVA performed to compare body weight of mice in each treatment condition revealed that while there was a main effect of genotype [$F(1,39) = 10.020$; $p = 0.003$] such that $Mc4r^{+/+}$ mice (22.71 ± 0.93 g) weighed less than $Mc4r^{-/-}$ mice (26.667 ± 0.84 g), there was no significant interaction between genotype and MTII dose, suggesting that body weight did not likely contribute to the genotype × MTII dose interaction effects observed with ethanol consumption and food intake data. Increased body weight in $Mc4r^{-/-}$ mice has previously been reported (Huszar et al., 1997; Marsh et al., 1999).

Experiment 3: Ethanol Consumption Following Peripheral Administration of the MCR Agonist MTII

Data showing 6-hour consumption measures following i.p. injection of MTII in the $Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice are presented in Fig. 2D–F. A two-way ANOVA performed on ethanol consumption data revealed a main effect of MTII

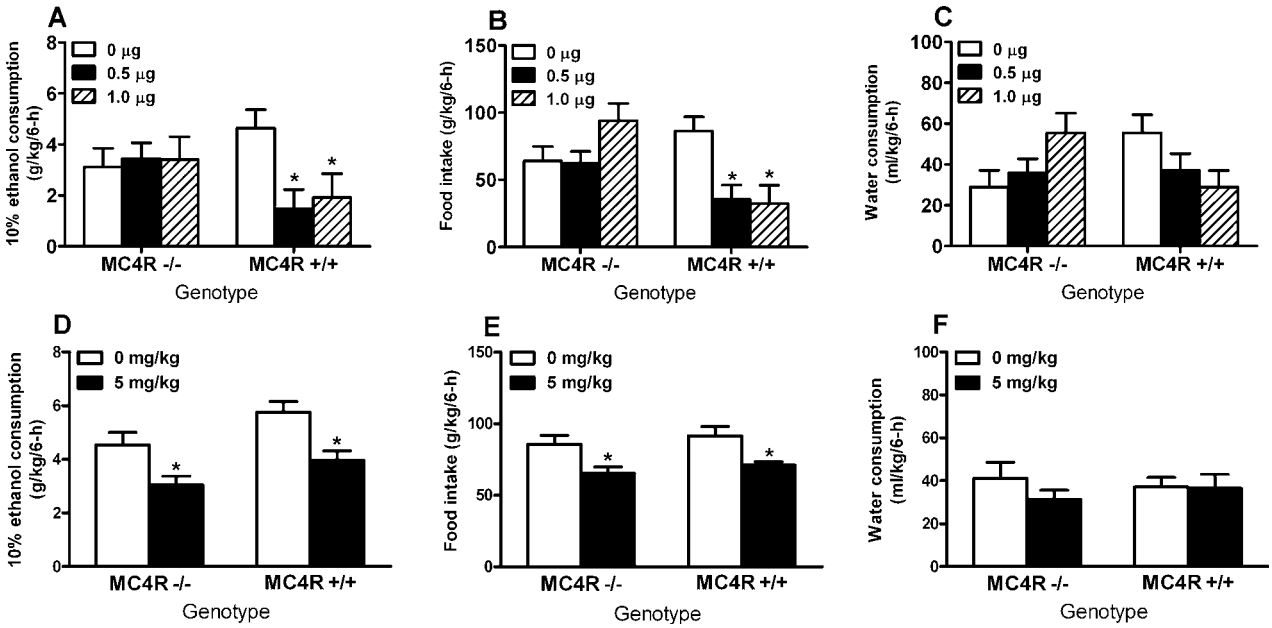

Fig. 2. Consumption of 10% (v/v) ethanol (g/kg/6-h), food (g/kg/6-h), and water (ml/kg/6-h) in $Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice given intracerebroventricular infusion of saline (0 μg) or melanotan-II (MTII) (0.5 or 1.0 μg) are presented in panels **A**, **B**, and **C**, respectively (Experiment 2). Similarly, consumption of ethanol, food, and water in $Mc4r^{-/-}$ and $Mc4r^{+/+}$ mice given intraperitoneal injection of 0.9% saline (0 mg/kg) or MTII (5 mg/kg) are presented in panels **D**, **E**, and **F**, respectively (Experiment 3). All values are means ± SEM. *$p < 0.05$ relative to 0 μg or 0 mg/kg dose.

dose [$F(1,55) = 17.22$; $p = 0.001$]. Neither the genotype main effect nor the interaction effect was significant (Fig. 2D). A two-way ANOVA performed on food intake data revealed a main effect of MTII dose [$F(1,55) = 14.423$; $p = 0.001$], but the genotype main effect and interaction effect did not achieve statistical significance (Fig. 2E). A two-way ANOVA performed on water intake data failed to show any statistically significant effects (Fig. 2F). Similarly, a two-way ANOVA performed on ethanol preference ratio data failed to show any significant effects (data not shown). Finally, a two-way ANOVA performed to compare body weight of mice in each treatment condition revealed a main effect of genotype [$F(1,55) = 7.023$; $p = 0.011$] such that $Mc4r^{+/+}$ mice (23.38 ± 0.67 g) weighed less than the $Mc4r^{-/-}$ mice (25.89 ± 0.66 g). No other effects were significant.

Experiments 4 and 5: Sucrose and Saccharin Solution Consumption Following Central Infusion of the MCR Agonist MTII

Figure 3 shows data representing 6-h consumption measures during sucrose testing in Experiment 4 (Fig. 3A–C) and saccharin testing in Experiment 5 (Fig. 3D–F) in C57BL/6J mice that were given i.c.v. infusion of vehicle or a 0.5-μg dose of MTII. One-way ANOVAs performed on sucrose, food, and water intake data from Experiment 4 revealed that the 0.5-μg dose of MTII significantly reduced sucrose [$F(1,13) = 8.477$; $p = 0.012$] and food [$F(1,13) = 6.456$; $p = 0.025$] intake but did not significantly alter water drinking relative to the control condition. One-way ANOVAs performed on saccharin, food, and water intake data from Experiment 5 revealed that the 0.5-μg dose of MTII significantly reduced saccharin intake relative to the control injection [$F(1,22) = 7.622$; $p = 0.011$], but did not significantly alter food or water intake.

DISCUSSION

Constitutive deletion of the MC4R was not associated with significant alterations of voluntary ethanol consumption or consumption of saccharin or sucrose solutions (Experiment 1). An initial conclusion might be that endogenous MC4R signaling does not play a critical role in modulating ethanol self-administration. However, developmental compensation in constitutive knockout mice may mask the contribution of the deleted gene (Gerlai, 1996, 2001); thus, a role for endogenous MC4R signaling in modulating ethanol drinking cannot be ruled out by null data. Interestingly, consistent with a recent report implicating MCR signaling in the modulation of water intake (Yosten and Samson, 2010), the present data suggest that endogenous MC4R signaling may play a role in the modulation of water intake as $Mc4r^{+/+}$ mice drank more water than $Mc4r^{-/-}$ mice over the course of Experiment 1. Importantly, i.c.v. infusion of MTII (0.5- and 1.0-μg doses) significantly reduced 6-hour ethanol consumption and food intake in $Mc4r^{+/+}$ mice without significantly altering water drinking, but failed to influence ethanol drinking or feeding in $Mc4r^{-/-}$ mice (Experiment 2). These observations support previous findings showing that MTII significantly reduces ethanol intake in C57BL/6J mice (Navarro et al., 2003, 2005) and extend the literature by showing that the MC4R is the primary receptor involved in

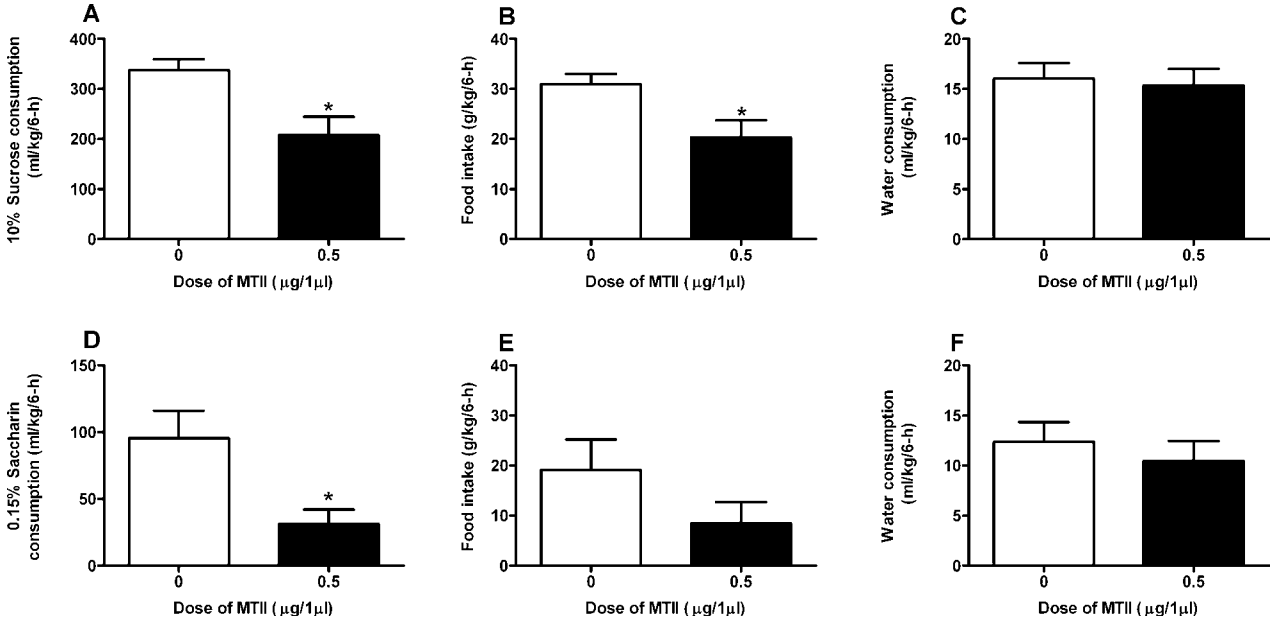

Fig. 3. Consumption of sweet solution (ml/kg/6-h; panels **A** and **D**), food (g/kg/6-h; panels **B** and **E**), and water (ml/kg/6-h; panels **C** and **F**) in C57BL/6J mice given intracerebroventricular infusion of saline (0 μg) or melanotan-II (0.5 μg). Sweet solution was made from 10% sucrose (Experiment 4; panels **A**, **B**, and **C**) or 0.15% saccharin (Experiment 4; panels **D**, **E**, and **F**). All values are means ± SEM. *$p < 0.05$ relative to 0-μg dose.

modulating the protective effects of centrally infused MTII on excessive ethanol intake. The present findings also replicate previous work demonstrating that central administration of MTII attenuates food intake (Grill et al., 1998; Hollopeter et al., 1998; Marsh et al., 1999; Navarro et al., 2003, 2005; Pierroz et al., 2002) and requires the MC4R (Marsh et al., 1999). Together, the present work highlights the critical role of the MC4R in modulating the central pharmacological effects of the MCR agonist MTII on ethanol intake and feeding. On the other hand, as Mc3r$^{-/-}$ mice showed normal ethanol drinking and food intake when MTII was centrally infused (Navarro et al., 2005), the MC3R does not appear to be involved.

Consistent with previous reports (Cettour-Rose and Rohner-Jeanrenaud, 2002; Navarro et al., 2003, 2005; Pierroz et al., 2002), here we show that the peripherally administered MTII (5 mg/kg) reduced ethanol drinking and food intake. However, unlike central administration, when administered peripherally, MTII did not require normal MC4R expression to suppress feeding or ethanol intake. This conclusion is supported by the observations that i.p. injection of MTII significantly reduced 6-hour ethanol consumption and food intake (but not water drinking) with similar effectiveness in Mc4r$^{-/-}$ and Mc4r$^{+/+}$ mice (Experiment 3). Because the MC4R is necessary for the central actions of MTII, the present data suggest that the effects of peripherally administered MTII on ethanol drinking and food intake may be modulated by other MCRs. A possibility is that peripheral MCRs (other than the MC4R) are involved. In fact, radiolabeled MTII, when given in an intravenous injection at a dose that attenuated food intake, was evident in the circumventricular organs but did not readily penetrate the blood–brain barrier in rats (Trivedi et al., 2003), and a more recent study showed low penetration of peripherally administered MTII into mouse brain (Hatziieremia et al., 2007). MC immunoreactivity and MC receptor binding have been observed in peripheral tissues, including the gastrointestinal tract and the adrenal glands (Dhillo et al., 2005; Saito et al., 1983; Tatro and Reichlin, 1987), and it is therefore possible that peripherally administered MTII attenuated ethanol consumption and food intake by actions within these peripheral regions. It should be noted that while a previous report showed that an i.p. injection of a 10 mg/kg dose of MTII reduced food intake in both Mc4r$^{-/-}$ and Mc4r$^{+/+}$ mice (Chen et al., 2000), a more recent finding showed that an i.p. injection of a 100-μg dose of MTII failed to alter feeding in Mc4r$^{-/-}$ mice but was effective in Mc4r$^{+/+}$ mice (Balthasar et al., 2005). Thus, it is also possible that lower doses of peripherally administered MTII require the MC4R to reduce food (and ethanol) intake, while higher doses (such as the 5 mg/kg dose used here) influence ingestive behaviors by acting on other MCRs. A more comprehensive assessment of the effects of peripherally administered MCR agonists, over a range of doses, on ethanol intake (as well as possible nonspecific effects) will be the focus of future research.

One goal of the present report was to assess the effects of MTII on the consumption of various reinforcing stimuli, in addition ethanol and food. I.c.v. infusion of a 0.5-μg dose of MTII, which significantly reduced 6-hour ethanol drinking and food intake in wild-type mice, also attenuated 6-hour consumption of a 10% sucrose solution and a 0.15% saccharin solution without altering water drinking. Thus, the MCR agonist MTII blunts the consumption of both caloric (ethanol, food, and sucrose) and noncaloric (saccharin) reinforcers, observations that are consistent with the hypothesis

that overlapping MC pathways modulate ethanol consumption and the consumption of natural reinforcers, regardless of caloric content. In fact, this should not come as a surprise in light of electrophysiological evidence demonstrating that both drugs of abuse and "natural" reinforcers (food and water) produce similar cell firing in the NAc (Carelli et al., 2000; Hollander et al., 2002; Roitman et al., 2004, 2005, 2008; Roop et al., 2002), and the observation that a growing list of peptides and proteins modulate both ethanol consumption and food intake (Thiele et al., 2003). For example, opioid receptor antagonists, which are approved for treating alcoholism, reduce both ethanol consumption and food intake (Gonzales and Weiss, 1998; Kamdar et al., 2007; Kotz et al., 1997; Middaugh et al., 2000; Yeomans and Gray, 2002). Interestingly, it has been proposed that cannabinoid receptor (CB1) agonists may be useful therapeutic agents for treating obesity (Cota et al., 2003) and alcoholism (Racz et al., 2003), and we suggest that MCR agonists may also provide a dual therapeutic role.

Given that administration of MTII was associated with reduced consumption of each of the reinforcing stimuli examined here, one potential concern is that administration of MTII produces nonspecific, and potentially aversive, effects. However, contrary to this hypothesis is the observation that MTII failed to significantly alter water intake relative to vehicle treatment in each of the experiments reported here, and we have previously observed MTII-induced attenuation of ethanol drinking that was not associated with altered water intake (Navarro et al., 2003, 2005). Another potential concern is that the effects of MCR agonists on ethanol drinking may be secondary to alterations of ethanol metabolism. However, this is unlikely because we have previously shown that peripheral administration and central administration of MTII do not alter blood ethanol clearance (Navarro et al., 2003, 2005).

Interestingly, while not significant when compared to the vehicle condition, there was a trend for the 1.0-μg dose of MTII to increase food intake in $Mc4r^{-/-}$ mice in Experiment 2. MCR agonist-induced increase in food intake has previously been reported in $Mc4r^{-/-}$ mice and was hypothesized to reflect a compensatory increase in MC3R signaling (Kumar et al., 2009). Consistent with this idea, a selective MC3R agonist was found to increase food intake, suggesting that the MC3R functions as a presynaptic autoreceptor in brain regions that modulate food intake (Cone, 2006; Marks et al., 2006).

In conclusion, the present work provides new insight into the mechanism by which MCR signaling influences ethanol consumption and feeding by demonstrating the essential role of central MC4R in modulating MCR agonist-induced reductions of ethanol intake and food intake. On the other hand, the MC4R does not modulate the effects of peripherally administered MCR agonist (MTII) on ethanol and food intake, suggesting that different populations of MCRs modulate the actions of centrally versus peripherally administered MTII. Centrally administered MTII also attenuated the consumption of sucrose and saccharin solutions at a dose that did not alter water drinking, consistent with the hypothesis that overlapping central MC pathways modulate the reinforcing properties of ethanol and natural reinforcers, independent of caloric content. Taken together, the present observations and previous work suggest that MC4R agonists, in addition to being attractive targets for treating obesity, may have therapeutic value for treating excessive ethanol consumption in individuals afflicted with alcohol abuse disorders or that are ethanol dependent.

ACKNOWLEDGMENTS

We thank Roger Cone for the generous donation of $MC4R^{-/-}$ mice, and Rhiannon Thomas for her assistance in collecting a portion of the behavioral data. This work was supported by NIH grants AA013573, AA015148, and AA017803, Department of Defense grants W81XWH-06-1-0158 and W81XWH-09-1-0293, and MEC grants (Spain) SEJ2006-03629, "Programa Salvador Madariaga 2006" and J.A., grant CTS1350.

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